

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



BZ

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/82, 15/29, 15/53, A01H 5/00, 5/10		A1	(11) International Publication Number: WO 98/45460 (43) International Publication Date: 15 October 1998 (15.10.98)
<p>(21) International Application Number: PCT/US98/07178</p> <p>(22) International Filing Date: 9 April 1998 (09.04.98)</p> <p>(30) Priority Data: 08/831,570 9 April 1997 (09.04.97) US</p> <p>(71) Applicant (<i>for all designated States except US</i>): RHONE-POULENC AGRO [FR/FR]; Dépt. Propriété Industrielle, 14-20, rue Pierre Balzat, F-69009 Lyon (FR).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (<i>for US only</i>): THOMAS, Terry, L. [US/US]; 2804 Cloister Drive, College Station, TX 77845 (US). BEREMAND, Phillip, D. [US/US]; 9208 Brookwater Circle, College Station, TX 77845 (US). NUNBERG, Andrew, N. [US/US]; 12215-B Encanto Lane, Maryland Heights, MO 63043 (US).</p> <p>(74) Agents: DiGIGLIO, Frank, S. et al.; Scully, Scott, Murphy & Presser, 400 Garden City Plaza, Garden City, NY 11530 (US).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p>(54) Title: A SUNFLOWER ALBUMIN 5' REGULATORY REGION FOR THE MODIFICATION OF PLANT SEED LIPID COMPOSITION</p> <p>(57) Abstract</p> <p>The present invention is directed to 5' regulatory regions of a sunflower albumin gene. The 5' regulatory regions, when operably linked to either the coding sequence of a heterologous gene or a sequence complementary to a native plant gene direct expression of the coding sequence or complementary sequence in a plant seed. The regulatory regions are useful in expression cassettes and expression vectors for the transformation of plants. Also provided are methods of modulating the levels of a heterologous gene or native plant gene such as a fatty acid synthesis or lipid metabolism gene by transforming a plant with the subject expression cassettes and expression vectors.</p>	

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

1 A SUNFLOWER ALBUMIN 5' REGULATORY REGION FOR THE
MODIFICATION OF PLANT SEED LIPID COMPOSITION

BACKGROUND OF THE INVENTION

5 Seed oil content has traditionally been modified by plant breeding. The use of recombinant DNA technology to alter seed oil composition can accelerate this process and in some cases alter seed oils in a way that cannot be accomplished by breeding
10 alone. The oil composition of *Brassica* has been significantly altered by modifying the expression of a number of lipid metabolism genes. Such manipulations of seed oil composition have focused on altering the proportion of endogenous component fatty acids. For example, antisense repression of the $\Delta 12$ -desaturase gene in transgenic rapeseed has resulted in an increase in oleic acid of up to 83%. Topfer et al.
15 1995 *Science* 268:681-686.

There have been some successful attempts at
20 modifying the composition of seed oil in transgenic plants by introducing new genes that allow the production of a fatty acid that the host plants were not previously capable of synthesizing. Van de Loo, et al. (1995 *Proc. Natl. Acad. Sci USA* 92:6743-6747) have been able to introduce a $\Delta 12$ -hydroxylase gene
25 into transgenic tobacco, resulting in the introduction of a novel fatty acid, ricinoleic acid, into its seed oil. The reported accumulation was modest from plants carrying constructs in which transcription of the
30 hydroxylase gene was under the control of the

- 2 -

1 cauliflower mosaic virus (CaMV) 35S promoter.

1 Similarly, tobacco plants have been engineered to produce low levels of petroselinic acid by expression of an acyl-ACP desaturase from coriander (Cahoon et al. 1992 *Proc. Natl. Acad. Sci USA* 89:11184-11188).

5 The long chain fatty acids (C18 and larger), have significant economic value both as nutritionally and medically important foods and as industrial commodities (Ohlrogge, J.B. 1994 *Plant Physiol.* 104:821-826). Linoleic (18:2 Δ9,12) and α-linolenic acid (18:3 Δ9,12,15) are essential fatty acids found in many seed oils. The levels of these fatty-acids have been manipulated in oil seed crops through breeding and biotechnology (Ohlrogge, et al. 1991 *Biochim. Biophys. Acta* 1082:1-26; Topfer et al. 1995 *Science* 268:681-686). Additionally, the production of novel fatty acids in seed oils can be of considerable use in both human health and industrial applications.

Consumption of plant oils rich in γ-linolenic acid (GLA) (18:3 Δ6,9,12) is thought to alleviate hypercholesterolemia and other related clinical disorders which correlate with susceptibility to coronary heart disease (Brenner R.R. 1976 *Adv. Exp. Med. Biol.* 83:85-101). The therapeutic benefits of dietary GLA may result from its role as a precursor to prostaglandin synthesis (Weete, J.D. 1980 in *Lipid Biochemistry of Fungi and Other Organisms*, eds. Plenum Press, New York, pp. 59-62). Linoleic acid(18:2) (LA) is transformed into gamma linolenic acid (18:3) (GLA) by the enzyme Δ6-desaturase.

30

- 3 -

Few seed oils contain GLA despite high
1 contents of the precursor linoleic acid. This is due
to the absence of $\Delta 6$ -desaturase activity in most
plants. For example, only borage (*Borago*
officinalis), evening primrose (*Oenothera biennis*),
5 and currants (*Ribes nigrum*) produce appreciable
amounts of linolenic acid. Of these three species,
only *Oenothera* and borage are cultivated as a
commercial source for GLA. It would be beneficial if
agronomic seed oils could be engineered to produce GLA
10 in significant quantities by introducing a
heterologous $\Delta 6$ -desaturase gene. It would also be
beneficial if other expression products associated
with fatty acid synthesis and lipid metabolism could
be produced in plants at high enough levels so that
15 commercial production of a particular expression
product becomes feasible.

As disclosed in U.S. Patent No. 5,552,306, a
cyanobacterial $\Delta 6$ -desaturase gene has been recently
isolated. Expression of this cyanobacterial gene in
20 transgenic tobacco resulted in significant but low
level GLA accumulation. (Reddy et al. 1996 *Nature*
Biotech. 14:639-642). Applicant's copending U.S.
Application Serial No. 08,366,779, discloses a $\Delta 6$ -
desaturase gene isolated from the plant *Borago*
25 *officinalis* and its expression in tobacco under the
control of the CaMV 35S promoter. Such expression
resulted in significant but low level GLA and
octadecatetraenoic acid (ODTA or OTA) accumulation in
seeds. Thus, a need exists for a promoter which
30 functions in plants and which consistently directs

- 4 -

high level expression of lipid metabolism genes in
1 transgenic plant seeds.

Sunflower embryos accumulate two major classes of storage proteins. These are the 11 S globulins, soluble in 1 M NaCl, and 2 S albumins,
5 soluble in water (Youle et al. 1981 *Am J. Bot* 68:44-48). The synthesis, processing and accumulation of 2 S albumin seed proteins have been studied intensively in *Brassica napus* (Crouch et al., 1983 *J. Mol. Appl. Genet.* 2:273-284; Ericson et al., 1986 *J. Biol. Chem.* 261:14576-14581), pea (Higgins et al., 1986 *Plant Mol. Biol.* 8:37-45), radish (Laroche-Raynal et al., 1986 *Eur. J. Biochem.* 157:321-327), castor bean (Lord J.M., 1985 *Eur. J. Biochem* 146:403-409) and Brazil nut (Sun et al., 1987 *Eur. J. Biochem* 162:477-483). A major
10 conclusion of these studies is that the characteristic low molecular weight, disulfide-linked albumin polypeptides found in mature seeds result from the extensive processing of larger precursors synthesized during embryogenesis. Two additional characteristics
15 that define the 2 S albumin seed storage proteins are high amide content and high frequency of cysteine residues (Youle et al., 1981).

In sunflower, the 2 S albumins represent more than 50% of the protein present in seeds (Youle et al., 1981) and consist of two or three closely related polypeptides with molecular weights of approximately 19 kDa (Cohen, E.A., 1986 "Analysis of sunflower 2S seed storage protein genes" MS thesis, Texas A&M University; Allen et al. 1987 *Plant Mol Biol* 5:165-173). The sunflower albumin is apparently

-5-

1 maintained in a compact structure by intramolecular
1 disulfide bonds resulting in a rapidly migrating
 species with an apparent molecular weight of 14 kDa
 when analyzed by SDS-polyacrylamide gel
 electrophoresis (SDS-PAGE) under non-reducing
5 conditions. When reduced, this species migrates as a
 19 kDa polypeptide (Cohen, E.A., 1986). In contrast,
 most other 2 S proteins are composed of large and
 small subunit polypeptides, derived from a single
 precursor, and linked by intermolecular disulfide bonds
10 (Crouch et al. 1983 *J. Mol. Appl. Genet.* 2:273-284;
 Ericson et al. 1986 *J. Biol. Chem.* 261:14576-14581;
 Sun et al. 1987, *Eur. J. Bioch.* 162:477-483.)

15 Albumin polypeptides can be detected in
 sunflower embryos by 5 days post-fertilization (DPF),
15 2 days before helianthinins are detectable, and
 continue to accumulate through seed maturation.
 Sunflower albumin mRNAs, also first detected at 5 DPF,
 accumulate rapidly in sunflower embryos reaching
 maximum prevalence between 12 and 15 DPF. After this
20 time albumin transcripts decrease in prevalence with
 kinetics similar to that observed for helianthinin
 mRNA (Allen et al. 1987). Functional sunflower
 albumin mRNAs are undetectable in dry seeds,
 germinated seedlings or leaves (Cohen 1986).

25 A number of albumin cDNAs and genomic clones
 have been isolated from different plant species
 including sunflower (Allen et al. 1987 *Mol-Gen Genet.*
 210:211-218) and pea (Higgins et al. 1986 *J. Biol.*
 Chem 261:11124-11130). As in other classes of seed
30 proteins such as *Brassica napis* (Crouch et al., 1983;

- 6 -

1 Ericson et al., 1986), 2 S albumin seed proteins are
1 encoded by small gene families.

5 The present invention provides 5' regulatory sequences from a sunflower albumin gene which direct high level expression of lipid metabolism genes in
5 transgenic plants. In accordance with the present invention, chimeric constructs comprising a sunflower albumin 5' regulatory region operably linked to coding sequence for a lipid metabolism gene such as a Δ6-
10 desaturase gene are provided. Transgenic plants comprising the subject chimeric constructs accumulate GLA to approximately 10% of C18 fatty acids. This is within the range of accumulation of GLA for *Oenothera biennis*, a primary commercial source for GLA.

15 **SUMMARY OF THE INVENTION**

The present invention is directed to 5' regulatory regions of a sunflower albumin gene. The 5' regulatory regions, when operably linked to either the coding sequence of a heterologous gene or sequence 20 complementary to a native plant gene, direct expression of the heterologous gene or complementary sequence in a plant seed.

The present invention thus provides expression cassettes and expression vectors comprising 25 an albumin 5' regulatory region operably linked to a heterologous gene or a sequence complementary to a native plant gene.

Plant transformation vectors comprising the expression cassettes and expression vectors are also 30 provided as are plant cells transformed by these

- 7 -

1 vectors, and plants and their progeny containing the
1 vectors.

In one embodiment of the invention, the heterologous gene or complementary sequence is a fatty acid synthesis gene or a lipid metabolism gene.

5 In another aspect of the present invention, a method is provided for producing a plant with increased levels of a product of a fatty acid synthesis or lipid metabolism gene.

10 In particular, there is provided a method for producing a plant with increased levels of a fatty acid synthesis or lipid metabolism gene by transforming a plant with the subject expression cassettes and expression vectors which comprise an albumin 5' regulatory region and a coding sequence for
15 a fatty acid synthesis or lipid metabolism gene.

20 In another aspect of the present invention, there is provided a method for cosuppressing a native fatty acid synthesis or lipid metabolism gene by transforming a plant with the subject expression cassettes and expression vectors which comprise an albumin 5' regulatory region and a coding sequence for
25 a fatty acid synthesis or lipid metabolism gene.

30 A further aspect of this invention provides a method of decreasing production of a native plant gene such as a fatty acid synthesis gene or a lipid metabolism gene by transforming a plant with an expression vector comprising an albumin 5' regulatory region operably linked to a nucleic acid sequence complementary to a native plant gene.

- 8 -

Also provided are methods of modulating the
1 levels of a heterologous gene or native plant gene
such as a fatty acid synthesis or lipid metabolism
gene by transforming a plant with the subject
expression cassettes and expression vectors.

5

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts the nucleotide and
corresponding amino acid sequence of the borage $\Delta 6$ -
desaturase gene (SEQ ID NO:1). The cytochrome b5
10 heme-binding motif is boxed and the putative metal
binding, histidine rich motifs (HRMs) are underlined.
The motifs recognized by the primers (PCR analysis)
are underlined with dotted lines, i.e. tgg aaa tgg aac
cat aa; and gag cat cat ttg ttt cc.

15 Fig. 2 is a dendrogram showing similarity of
the borage $\Delta 6$ -desaturase to other membrane-bound
desaturases. The amino acid sequence of the borage $\Delta 6$ -
desaturase was compared to other known desaturases
using Gene Works (IntelliGenetics). Numerical values
20 correlate to relative phylogenetic distances between
subgroups compared.

Fig. 3A provides a gas liquid chromatography
profile of the fatty acid methyl esters (FAMES)
derived from leaf tissue of a wild type tobacco
25 'Xanthi'.

Fig. 3B provides a gas liquid chromatography
profile of the FAMES derived from leaf tissue of a
tobacco plant transformed with the borage $\Delta 6$ -
desaturase cDNA under transcriptional control of the
30 CaMV 35S promoter (pAN2). Peaks corresponding to

- 9 -

1 methyl linoleate (18:2), methyl γ -linolenate (18:3 γ),
1 methyl α -linolenate (18:3 α), and methyl
octadecatetraenoate (18:4) are indicated.

5 Fig. 4 is the nucleotide sequence of the
HaG5 regulatory region. The transcriptional start
site (+1) is indicated by a bold T. The underlined
Bam HI restriction site was introduced by PCR.

Fig. 5 is a scheme depicting construction of
the sunflower albumin HaG5 regulatory region/ Δ 6-
desaturase gene expression vector.

10 Fig. 6A is an RNA gel blot analysis carried
out on 5 μ g samples of RNA isolated from borage leaf,
root, and 12 dpp embryo tissue, using labeled borage
 Δ 6-desaturase cDNA as a hybridization probe.

15 Fig. 6B depicts a graph corresponding to the
Northern analysis results for the experiment shown in
Fig. 6A.

Fig. 7 is a PCR analysis showing the
presence of the borage delta 6-desaturase gene in
transformed plants of oilseed rape. Lanes 1,3 and 4
20 were loaded with PCR reactions performed with DNA from
plants transformed with the borage delta 6-desaturase
gene linked to the oleosin 5' regulatory region; lane
2: DNA from plant transformed with the borage delta
6-desaturase gene linked to the albumin 5' regulatory
25 region; lanes 5 and 6: DNA from non-transformed
plants; lane 7: molecular weight marker (1 kb ladder,
Gibco BRL); lane 8: PCR without added template DNA;
lane 9: control with DNA from *Agrobacterium*
tumefaciens EHA 105 containing the plasmid pAN3 the

30

-10-

borage delta 6-desaturase gene linked to the oleosin
1 5' regulatory region.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides isolated
5 nucleic acids encoding 5' regulatory regions from a
sunflower albumin gene. In accordance with the
present invention, the subject 5' regulatory regions,
when operably linked to either a coding sequence of a
heterologous gene or sequence complementary to a
10 native plant gene, direct expression of the coding
sequence or the complementary sequence in a plant
seed. The albumin 5' regulatory regions of the
present invention are useful in the construction of an
expression cassette which comprises in the 5' to 3'
15 direction, a subject albumin 5' regulatory region, a
heterologous gene or sequence complementary to a
native plant gene under control of the regulatory
region and a 3' termination sequence. Such an
expression cassette can be incorporated into a variety
20 of autonomously replicating vectors in order to
construct an expression vector.

In accordance with the present invention, it
has been surprisingly found that plants transformed
with a subject expression vector accumulate GLA to
25 approximately 10% of C18 fatty acids. Such an
accumulation is within the range of accumulation of
GLA for *Oenothera biennis*, a primary commercial source
for GLA.

As used herein, the term "cassette" refers
30 to a nucleotide sequence capable of expressing a

-11-

particular gene if said gene is inserted so as to be
1 operably linked to one or more regulatory regions
present in the nucleotide sequence. Thus, for
example, the expression cassette may comprise a
5 heterologous coding sequence which is desired to be
expressed in a plant seed. The expression cassettes
and expression vectors of the present invention are
therefore useful for directing seed-specific
expression of any number of heterologous genes. The
term "seed-specific expression" as used herein, refers
10 to expression in various portions of a plant seed such
as the endosperm and embryo.

An isolated nucleic acid encoding a 5'
regulatory region from a sunflower albumin gene can be
provided as follows. Albumin recombinant genomic
15 clones are isolated by screening a sunflower genomic
DNA library with a cDNA (or a portion thereof)
representing albumin mRNA. A number of different
albumin cDNAs have been isolated. The methods used to
isolate such cDNAs as well as the nucleotide and
20 corresponding amino acid sequences have been
published. Higgins et al., 1986 *J. Biol. Chem.* 261:
11124-11130; Allen et al., 1987 in *Molecular
Approaches to Developmental Biology*, Alan R. Liss,
Inc., pp. 415-424.

25 Methods considered useful in obtaining
albumin genomic recombinant DNA are provided in
Sambrook et al. 1989, in *Molecular Cloning: A
Laboratory Manual*, Cold Spring Harbor, NY, for
example, or any of the myriad of laboratory manuals on
30 recombinant DNA technology that are widely available.

- 12 -

To determine nucleotide sequences, a multitude of
1 techniques are available and known to the ordinarily
skilled artisan. For example, restriction fragments
containing an albumin regulatory region can be
subcloned into the polylinker site of a sequencing
5 vector such as pBluescript (Stratagene). These
pBluescript subclones can then be sequenced by the
double-stranded dideoxy method (Chen and Seeburg,
1985, *DNA* 4:165).

In a preferred embodiment, the sunflower
10 albumin regulatory region comprises nucleotides 860 to
+29 of Fig. 4 (nucleotides 1-895 of SEQ ID NO:2).
Modifications to the albumin regulatory region as set
forth in SEQ ID NO:2 which maintain the characteristic
property of directing seed-specific expression, are
15 within the scope of the present invention. Such
modifications include insertions, deletions and
substitutions of one or more nucleotides.

The 5' regulatory region of the present
invention can be derived from restriction endonuclease
20 or exonuclease digestion of an albumin genomic clone.
Thus, for example, the known nucleotide or amino acid
sequence of the coding region of an isolated albumin
gene is aligned to the nucleic acid or deduced amino
acid sequence of an isolated albumin genomic clone and
25 5' flanking sequence (i.e., sequence upstream from the
translational start codon of the coding region) of the
isolated albumin genomic clone located.

The albumin 5' regulatory region as set
forth in SEQ ID NO:2 (nucleotides -860 to +29 of Fig.
30 4) may be generated from a genomic clone having either

- 13 -

or both excess 5' flanking sequence or coding sequence
1 by exonuclease III-mediated deletion. This is
accomplished by digesting appropriately prepared DNA
with exonuclease III (exoIII) and removing aliquots at
increasing intervals of time during the digestion.
5 The resulting successively smaller fragments of DNA
may be sequenced to determine the exact endpoint of
the deletions. There are several commercially
available systems which use exonuclease III (exoIII)
to create such a deletion series, e.g. Promega
10 Biotech, "Erase-A-Base" system. Alternatively, PCR
primers can be defined to allow direct amplification
of the subject 5' regulatory regions.

Using the same methodologies, the
ordinarily skilled artisan can generate one or more
15 deletion fragments of nucleotides 1-895 as set forth
in SEQ ID NO:2. Any and all deletion fragments which
comprise a contiguous portion of nucleotides set forth
in SEQ ID NO:2 and which retain the capacity to direct
seed-specific expression are contemplated by the
20 present invention.

The identification of albumin 5' regulatory
sequences which direct seed-specific expression
comprising nucleotides 1-895 of SEQ ID NO:2 and
modifications or deletion fragments thereof, can be
25 accomplished by transcriptional fusions of specific
sequences with the coding sequences of a heterologous
gene, transfer of the chimeric gene into an
appropriate host, and detection of the expression of
the heterologous gene. The assay used to detect
30 expression depends upon the nature of the heterologous

-14-

sequence. For example, reporter genes, exemplified by
1 chloramphenicol acetyl transferase and β -glucuronidase (GUS), are commonly used to assess transcriptional and translational competence of chimeric constructions.
Standard assays are available to sensitively detect
5 the reporter enzyme in a transgenic organism. The β -glucuronidase (GUS) gene is useful as a reporter of promoter activity in transgenic plants because of the high stability of the enzyme in plant cells, the lack of intrinsic β -glucuronidase activity in higher plants
10 and availability of a quantitative fluorimetric assay and a histochemical localization technique.
Jefferson et al. (1987 *EMBO J* 6:3901) have established standard procedures for biochemical and histochemical detection of GUS activity in plant
15 tissues. Biochemical assays are performed by mixing plant tissue lysates with 4-methylumbelliferyl- β -D-glucuronide, a fluorimetric substrate for GUS, incubating one hour at 37°C, and then measuring the fluorescence of the resulting 4-methyl-umbelliferone.
20 Histochemical localization for GUS activity is determined by incubating plant tissue samples in 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc) for about 18 hours at 37°C and observing the staining pattern of X-Gluc. The construction of such chimeric
25 genes allows definition of specific regulatory sequences and demonstrates that these sequences can direct expression of heterologous genes in a seed-specific manner.

Another aspect of the invention is directed
30 to a chimeric plant gene comprising a 5' regulatory

-15-

1 region from an albumin gene which directs seed
specific expression operably linked to the coding
sequence of a heterologous gene such that the
regulatory element is capable of controlling
expression of the product encoded by the heterologous
5 gene. The heterologous gene can be any gene other
than albumin. If necessary, additional regulatory
elements or parts of these elements sufficient to
cause expression resulting in production of an
effective amount of the polypeptide encoded by the
10 heterologous gene are included in the chimeric
constructs.

Accordingly, the present invention provides
chimeric genes comprising sequences of the albumin 5'
regulatory region that confer seed-specific expression
15 which are operably linked to a sequence encoding a
heterologous gene such as a lipid metabolism enzyme.
Examples of lipid metabolism and fatty acid synthesis
genes useful for practicing the present invention
include lipid desaturases such as $\Delta 6$ -desaturases, $\Delta 12$ -
20 desaturases, $\Delta 15$ -desaturases and other related
desaturases such as stearoyl-ACP desaturases, acyl
carrier proteins (ACPs), thioesterases, acetyl
transacylases, acetyl-coA carboxylases, ketoacyl-
synthases, malonyl transacylases, and elongases. Such
25 lipid metabolism and fatty acid synthesis genes have
been isolated and characterized from a number of
different bacteria and plant species. Their
nucleotide coding sequences as well as methods of
isolating such coding sequences are disclosed in the
30

-16-

published literature and are widely available to those
1 of skill in the art.

In particular, the Δ6-desaturase genes
disclosed in U.S. Patent No. 5,552,306 and
applicants' copending U.S. Application Serial No.
5 08/366,779 filed December 30, 1994 and incorporated
herein by reference, are contemplated as lipid
metabolism genes particularly useful in the practice
of the present invention.

The chimeric genes of the present invention
10 are constructed by ligating a 5' regulatory region of
an albumin genomic DNA to the coding sequence of a
heterologous gene. The juxtaposition of these
sequences can be accomplished in a variety of ways.
In a preferred embodiment the order of the sequences,
15 from 5' to 3', is an albumin 5' regulatory region
(including a promoter), a coding sequence, and a
termination sequence which includes a polyadenylation
site.

Standard techniques for construction of such
20 chimeric genes are well known to those of ordinary
skill in the art and can be found in references such
as Sambrook et al. (1989). A variety of strategies are
available for ligating fragments of DNA, the choice of
which depends on the nature of the termini of the DNA
25 fragments. One of ordinary skill in the art
recognizes that in order for the heterologous gene to
be expressed, the construction requires promoter
elements and signals for efficient polyadenylation of
the transcript. Accordingly, the albumin 5'
30 regulatory region that contains the consensus promoter

-17-

sequence known as the TATA box can be ligated directly
1 to a promoterless heterologous coding sequence.

The restriction or deletion fragments that contain the albumin TATA box are ligated in a forward orientation to a promoterless heterologous gene such
5 as the coding sequence of β -glucuronidase (GUS). The skilled artisan will recognize that the subject albumin 5' regulatory regions can be provided by other means, for example chemical or enzymatic synthesis.
The 3' end of a heterologous coding sequence is
10 optionally ligated to a termination sequence comprising a polyadenylation site, exemplified by, but not limited to, the nopaline synthase polyadenylation site, or the octopine T-DNA gene 7 polyadenylation site. Alternatively, the polyadenylation site can be
15 provided by the heterologous gene.

The present invention also provides methods of increasing levels of heterologous genes in plant seeds. In accordance with such methods, the subject expression cassettes and expression vectors are
20 introduced into a plant in order to effect expression of a heterologous gene. For example, a method of producing a plant with increased levels of a product of a fatty acid synthase or lipid metabolism gene is provided by transforming a plant cell with an
25 expression vector comprising an albumin 5' regulatory region operably linked to a fatty acid synthesis or lipid metabolism gene and regenerating a plant with increased levels of the product of said fatty acid synthesis or lipid metabolism gene.

30

-18-

Another aspect of the present invention
1 provides methods of reducing levels of a product of a
gene which is native to a plant which comprises
transforming a plant cell with an expression vector
comprising a subject albumin regulatory region
5 operably linked to a nucleic acid sequence which is
complementary to the native plant gene. In this
manner, levels of endogenous product of the native
plant gene are reduced through the mechanism known as
10 antisense regulation. Thus, for example, levels of a
product of a fatty acid synthesis gene or lipid
metabolism gene are reduced by transforming a plant
with an expression vector comprising a subject albumin
5' regulatory region operably linked to a nucleic acid
sequence which is complementary to a nucleic acid
15 sequence coding for a fatty acid synthesis or lipid
metabolism gene.

The present invention also provides a method
of cosuppressing a gene which is native to a plant
which comprises transforming a plant cell with an
20 expression vector comprising a subject albumin
regulatory region operably linked to a nucleic acid
sequence coding for the native plant gene. In this
manner, levels of endogenous product of the native
plant gene are reduced through the mechanism known as
25 cosuppression. Thus, for example, levels of a product
of a fatty acid synthesis gene or lipid metabolism
gene are reduced by transforming a plant with an
expression vector comprising a subject albumin 5'
regulatory region operably linked to a nucleic acid
30 sequence coding for a fatty acid synthesis or lipid

-19-

metabolism gene native to the plant. Although the
1 exact mechanism of cosuppression is not completely
understood, one skilled in the art is familiar with
published works reporting the experimental conditions
and results associated with cosuppression (Napoli et
5 al. 1990 *The Plant Cell* 2:270-289; Van der Krol 1990
The Plant Cell 2:291-299.

To provide regulated expression of the
heterologous or native genes, plants are transformed
with the chimeric gene constructions of the invention.
10 Methods of gene transfer are well known in the art.
The chimeric genes can be introduced into plants by
leaf disk transformation-regeneration procedure as
described by Horsch et al. (1985) *Science* 227:1229.
Other methods of transformation such as protoplast
15 culture (Horsch et al. 1984 *Science* 223:496, DeBlock
et al. 1984 *EMBO J.* 2:2143, Barton et al. 1983, *Cell*
32:1033) can also be used and are within the scope of
this invention. In a preferred embodiment, plants are
transformed with *Agrobacterium*-derived vectors such as
20 those described in Klett et al. (1987) *Annu. Rev.*
Plant Physiol. 38:467. Other well-known methods are
available to insert the chimeric genes of the present
invention into plant cells. Such alternative methods
include biolistic approaches (Klein et al. 1987 *Nature*
25 327:70), electroporation, chemically-induced DNA
uptake, and use of viruses or pollen as vectors.

When necessary for the transformation
method, the chimeric genes of the present invention
can be inserted into a plant transformation vector,
30 e.g. the binary vector described by Bevan, M. 1984

- 20 -

Nucl. Acids Res. 12:8711-8721. Plant transformation
1 vectors can be derived by modifying the natural gene
transfer system of *Agrobacterium tumefaciens*. The
natural system comprises large Ti (tumor-inducing)-
plasmids containing a large segment, known as T-DNA,
5 which is transferred to transformed plants. Another
segment of the Ti plasmid, the vir region, is
responsible for T-DNA transfer. The T-DNA region is
bordered by terminal repeats. In the modified binary
vectors, the tumor inducing genes have been deleted
10 and the functions of the vir region are utilized to
transfer foreign DNA bordered by the T-DNA border
sequences. The T-region also contains a selectable
marker for antibiotic resistance, and a multiple
cloning site for inserting sequences for transfer.
15 Such engineered strains are known as "disarmed" *A.*
tumefaciens strains, and allow the efficient transfer
of sequences bordered by the T-region into the nuclear
genome of plants.

Surface-sterilized leaf disks or other
20 susceptible tissues are inoculated with the "disarmed"
foreign DNA-containing *A. tumefaciens*, cultured for a
number of days, and then transferred to antibiotic-
containing medium. Transformed shoots are then
selected after rooting in medium containing the
25 appropriate antibiotic, and transferred to soil.
Transgenic plants are pollinated and seeds from these
plants are collected and grown on antibiotic medium.

Expression of a heterologous or reporter
gene in developing seeds, young seedlings and mature
30 plants can be monitored by immunological,

- 21 -

histochemical or activity assays. As discussed herein,
1 the choice of an assay for expression of the chimeric
gene depends upon the nature of the heterologous
coding region. For example, Northern analysis can be
used to assess transcription if appropriate nucleotide
5 probes are available. If antibodies to the
polypeptide encoded by the heterologous gene are
available, Western analysis and immunohistochemical
localization can be used to assess the production and
localization of the polypeptide. Depending upon the
10 heterologous gene, appropriate biochemical assays can
be used. For example, acetyltransferases are detected
by measuring acetylation of a standard substrate. The
expression of a lipid desaturase gene can be assayed
by analysis of fatty acid methyl esters (FAMES).

15 Another aspect of the present invention
provides transgenic plants or progeny of these plants
containing the chimeric genes of the invention. Both
monocotyledonous and dicotyledonous plants are
contemplated. Plant cells are transformed with the
20 chimeric genes by any of the plant transformation
methods described above. The transformed plant cell,
usually in the form of a callus culture, leaf disk or
whole plant (via the vacuum infiltration method of
Bechtold et al. 1993 *C.R. Acad. Sci. Paris*, 316:1194-
25 1199) is regenerated into a complete transgenic plant
by methods well-known to one of ordinary skill in the
art (e.g. Horsh et al. 1985 *Science* 227:1129). In a
preferred embodiment, the transgenic plant is
sunflower, cotton, oil seed rape, maize, tobacco,
30 *Arabidopsis*, peanut or soybean. Since progeny of

- 22 -

1 transformed plants inherit the chimeric genes, seeds
or cuttings from transformed plants are used to
maintain the transgenic line.

The following examples further illustrate
the invention.

5

10

15

20

25

30

35

-23-

EXAMPLE 1

1 **Isolation of Membrane-Bound Polysomal
RNA and Construction of Borage cDNA Library**

5 Membrane-bound polysomes were isolated from
borage seeds 12 days post pollination (12 DPP) using
the protocol established for peas by Larkins and
Davies (1975 *Plant Phys.* 55: 749-756). RNA was
extracted from the polysomes as described by Mechler
10 (1987 *Methods in Enzymology* 152: 241-248, Academic
Press). Poly-A⁺ RNA was isolated from the membrane
bound polysomal RNA using Oligotex-dT™ beads (Qiagen).

Corresponding cDNA was made using
Stratagene's ZAP cDNA synthesis kit. The cDNA library
15 was constructed in the lambda ZAP II vector
(Stratagene) using the lambda ZAP II kit. The primary
library was packaged with Gigapack II Gold packaging
extract (Stratagene).

20

25

30

35

-24-

EXAMPLE 2

1 Isolation of a Δ-6 Desaturase cDNA from Borage

Hybridization protocol

5 The amplified borage cDNA library was plated at low density (500 pfu on 150 mm petri dishes). Highly prevalent seed storage protein cDNAs were reduced (subtracted from the total cDNAs) by screening with the corresponding cDNAs.

10 Hybridization probes for screening the borage cDNA library were generated by using random primed DNA synthesis as described by Ausubel *et al* (1994 Current Protocols in Molecular Biology, Wiley Interscience, N.Y.) and corresponded to previously identified abundantly expressed seed storage protein cDNAs. Unincorporated nucleotides were removed by use of a G-50 spin column (Boehringer Manheim). Probe was denatured for hybridization by boiling in a water bath for 5 minutes, then quickly cooled on ice.

15 Nitrocellulose filters carrying fixed recombinant bacteriophage were prehybridized at 60°C for 2-4 hours in hybridization solution [4X SET (600 mM NaCl, 80 mM Tris-HCl, 4 mM Na₂EDTA; pH 7.8), 5X Denhardt's reagent (0.1% bovine serum albumin, 0.1% Ficoll, and 0.1% polyvinylpyrrolidone), 100 µg/ml denatured salmon sperm DNA, 50 µg/ml polyadenine and 10 ug/ml polycytidine]. This was replaced with fresh hybridization solution to which denatured radioactive probe (2 ng/ml hybridization solution) was added. The filters were

20 incubated at 60°C with agitation overnight. Filters

25

30

-25-

were washed sequentially in 4X, 2X, and 1X SET (150 mM
1 NaCl, 20 mM Tris-HCl, 1 mM Na₂EDTA; pH7.8) for 15
minutes each at 60°C. Filters were air dried and then
exposed to X-ray film for 24 hours with intensifying
screens at -80°C.

5 Non-hybridizing plaques were excised using
Stratagene's excision protocol and reagents.
Resulting bacterial colonies were used to inoculate
liquid cultures and were either sequenced manually or
by an ABI automated sequencer.

10

Random Sequencing of cDNAs from a Borage Seed 12 (DPP)
Membrane-Bound Polysomal Library

Each cDNA corresponding to a non-
hybridizing plaque was sequenced once and a sequence
tag generated from 200-300 base pairs. All sequencing
15 was performed by cycle sequencing (Epicentre). Over
300 expressed sequence tags (ESTs) were generated.
Each sequence tag was compared to GenBank database
using the BLAST algorithm (Altschul et al. 1990 J.
20 Mol. Biol. 215:403-410). A number of lipid metabolism
genes, including the Δ6-desaturase were identified.

Database searches with the cDNA clone
designated mbp-65 using BLASTX with the GenBank
database resulted in a significant match to the
previously isolated *Synechocystis* Δ6-desaturase. It
25 was determined however, that mbp-65 was not a full
length cDNA. A full length cDNA was isolated using
mbp-65 to screen the borage membrane-bound polysomal
library. The resultant clone was designated pAN1 and
30 the cDNA insert of pAN1 was sequenced by the cycle

- 26 -

sequencing method. The amino acid sequence deduced
1 from the open reading frame (Fig. 1, SEQ ID NO:1) was
compared to other known desaturases using Geneworks
(IntelliGenetics) protein alignment program. This
alignment indicated that the cDNA insert of pAN1 was
5 the borage Δ^6 -desaturase gene.

The resulting dendrogram (Figure 2) shows
that Δ^{15} -desaturases and Δ^{12} -desaturases comprise two
groups. The newly isolated borage sequence and the
previously isolated *Synechocystis* Δ^6 -desaturase (U.S.
10 Patent No. 5,552,306) formed a third distinct group.
A comparison of amino acid motifs common to
desaturases and thought to be involved catalytically
in metal binding illustrates the overall similarity of
the protein encoded by the borage gene to desaturases
15 in general and the *Synechocystis* Δ^6 -desaturase in
particular (Table 1). At the same time, comparison of
the motifs in Table 1 indicates definite differences
between this protein and other plant desaturases.
Furthermore, the borage sequence is also distinguished
20 from known plant membrane associated fatty acid
desaturases by the presence of a heme binding motif
conserved in cytochrome b₅ proteins (Schmidt et al.
1994 *Plant Mol. Biol.* 26:631-642) (Figure 1). Thus,
while these results clearly suggested that the
25 isolated cDNA was a borage Δ^6 -desaturase gene, further
confirmation was necessary. To confirm the identity
of the borage Δ^6 -desaturase cDNA, the cDNA insert from
pAN1 was cloned into an expression cassette for stable
expression. The vector pBI121 (Jefferson et al. 1987
30 *EMBO J.* 6:3901-3907) was prepared for ligation by

-27-

digestion with BamHI and EcoICR I (an isoschizomer of
1 SacI which leaves blunt ends; available from Promega)
which excises the GUS coding region leaving the 35S
promoter and NOS terminator intact. The borage Δ^6 -
desaturase cDNA was excised from the recombinant
5 plasmid (pAN1) by digestion with BamHI and XhoI. The
XhoI end was made blunt by performing a fill-in
reaction catalyzed by the Klenow fragment of DNA
polymerase I. This fragment was then cloned into the
BamHI/EcoICR I sites of pBI121.1, resulting in the
10 plasmid pAN2.

15

20

25

30

35

1
5
10
15
20
25
30

35

TABLE 1
COMPARISON OF COMMON AMINO ACID MOTIFS IN MEMBRANE-BOUND DESATURASES

<u>Desaturase</u>	<u>Lipid Box</u>	<u>Metal Box 1</u>	<u>Metal Box 2</u>
Borage Δ^6	VIGHDAGH (SEQ. ID. NO:3)	HNAHH (SEQ. ID. NO:9)	FQLEHH (SEQ. ID. NO:17)
Synechocystis Δ^6	VGHDANH (SEQ. ID. NO:4)	HNYLHH (SEQ. ID. NO:10)	HQVTHH (SEQ. ID. NO:18)
Arab. chloroplast Δ^{15}	VLGHDCGH (SEQ. ID. NO:5)	HRTHH (SEQ. ID. NO:11)	HVTHH (SEQ. ID. NO:19)
Rice Δ^{15}	VLGHDCGH (SEQ. ID. NO:5)	HRTHH (SEQ. ID. NO:11)	HVTHH (SEQ. ID. NO:19)
Glycine chloroplast Δ^{15}	VLGHDCGH (SEQ. ID. NO:5)	HRTHH (SEQ. ID. NO:11)	HVTHH (SEQ. ID. NO:19)
Arab. fad3 (Δ^{15})	VLGHDCGH (SEQ. ID. NO:5)	HRTHH (SEQ. ID. NO:11)	HVTHH (SEQ. ID. NO:19)
Brassica fad 3 (Δ^{15})	VLGHDCGH (SEQ. ID. NO:5)	HRTHH (SEQ. ID. NO:11)	HVTHH (SEQ. ID. NO:19)
Borage Δ^{13} (P1-81)*	VIAHECGH (SEQ. ID. NO:6)	HRRHH (SEQ. ID. NO:12)	HVAHH (SEQ. ID. NO:20)
Arab. fad2 (Δ^{13})	VIAHECGH (SEQ. ID. NO:6)	HRRHH (SEQ. ID. NO:12)	HVAHH (SEQ. ID. NO:20)
Arab. chloroplast Δ^{13}	VIGHDCAH (SEQ. ID. NO:7)	HDRHH (SEQ. ID. NO:13)	HIPHH (SEQ. ID. NO:21)
Glycine plastid Δ^{12}	VIGHDCAH (SEQ. ID. NO:7)	HDRHH (SEQ. ID. NO:13)	HIPHH (SEQ. ID. NO:21)
Spinach plastidial n-6	VIGHDCAH (SEQ. ID. NO:7)	HDQHH (SEQ. ID. NO:14)	HIPHH (SEQ. ID. NO:21)
Synechocystis Δ^{12}	VVGHDCCGH (SEQ. ID. NO:8)	HDHHH (SEQ. ID. NO:15)	HIPHH (SEQ. ID. NO:21)
Anabaena Δ^{12}	VLGHDCGH (SEQ. ID. NO:5)	HNNHHH (SEQ. ID. NO:16)	HVPHH (SEQ. ID. NO:22)

*P1-81 is a full length cDNA which was identified by EST analysis and shows high similarity to the Arabidopsis Δ^{12} desaturase (fad2)

-29-

EXAMPLE 3

1

Production of Transgenic
Plants and Preparation and
Analysis of Fatty Acid Methyl Esters (FAMEs)

5 The expression plasmid, pAN2 was used to transform tobacco (*Nicotiana tabacum* cv. *xanthi*) via *Agrobacterium tumefaciens* according to standard procedures (Horsch, et al. 1985 *Science* 227:1229-1231; Bogue et al. 1990 *Mol. Gen. Genet.* 221:49-57) except
10 that the initial transformants were selected on 100 µg/ml kanamycin.

Tissue from transgenic plants was frozen in liquid nitrogen and lyophilized overnight. FAMEs were prepared as described by Dahmer, et al. (1989) *J. Amer. Oil. Chem. Soc.* 66: 543-548. In some cases, the solvent was evaporated again, and the FAMEs were resuspended in ethyl acetate and extracted once with deionized water to remove any water soluble contaminants. FAMEs were analyzed using a Tracor-560
20 gas liquid chromatograph as previously described (Reddy et al. 1996 *Nature Biotech.* 14:639-642).

As shown in Figure. 3, transgenic tobacco leaves containing the borage cDNA produced both GLA and octadecatetraenoic acid (OTA) (18:4 Δ6,9,12,15).
25 These results thus demonstrate that the isolated cDNA encodes a borage Δ6-desaturase.

30

35

- 30 -

EXAMPLE 4

1 Expression of $\Delta 6$ -desaturase in Borage

The native expression of $\Delta 6$ -desaturase was examined by Northern Analysis of RNA derived from 5 borage tissues. RNA was isolated from developing borage embryos following the method of Chang et al. 1993 *Plant Mol. Biol. Rep.* 11:113-116. RNA was electrophoretically separated on formaldehyde-agarose gels, blotted to nylon membranes by capillary 10 transfer, and immobilized by baking at 80°C for 30 minutes following standard protocols (Brown T., 1996 in *Current Protocols in Molecular Biology*, eds. Auselbel, et al. [Greene Publishing and Wiley-Interscience, New York] pp. 4.9.1-4.9.14.). The 15 filters were preincubated at 42°C in a solution containing 50% deionized formamide, 5X Denhardt's reagent, 5X SSPE (900 mM NaCl; 50mM Sodium phosphate, pH7.7; and 5 mM EDTA), 0.1% SDS, and 200 μ g/ml denatured salmon sperm DNA. After two hours, the 20 filters were added to a fresh solution of the same composition with the addition of denatured radioactive hybridization probe. In this instance, the probes used were borage legumin cDNA (Fig. 16A), borage oleosin cDNA (Fig. 16B), and borage $\Delta 6$ -desaturase cDNA 25 (pAN1, Example 2) (Fig. 16C). The borage legumin and oleosin cDNAs were isolated by EST cloning and identified by comparison to the GenBank database using the BLAST algorithm as described in Example 2. Loading variation was corrected by normalizing to 30 levels of borage EF1 α mRNA. EF1 α mRNA was identified

-31-

1 by correlating to the corresponding cDNA obtained by
the EST analysis described in Example 2. The filters
were hybridized at 42°C for 12-20 hours, then washed
as described above (except that the temperature was
65°C), air dried, and exposed to X-ray film.

5 As depicted in Figs. 15A and 15B, Δ6-
desaturase is expressed primarily in borage seed.
Borage seeds reach maturation between 18-20 days post
pollination (dpp). Δ6-desaturase mRNA expression
occurs throughout the time points collected (8-20
10 dpp), but appears maximal from 10-16 days post
pollination. This expression profile is similar to
that seen for borage oleosin and 12S seed storage
protein mRNAs (Figs. 16A, 16B, and 16C).

15

20

25

30

35

- 32 -

1

EXAMPLE 5

ISOLATION OF A SUNFLOWER ALBUMIN cDNA

5 The sunflower albumin cDNA (Ha5) was
isolated by differentially screening a sunflower cDNA
library using cDNA probes from leaf and 12 DPF (days
post flowering) embryos. A cDNA of 1011 bp was
obtained (Cohen E.A. "Analysis of sunflower 2S seed
storage protein genes" MS thesis, Texas A&M
10 University, Allen et al., 1987a in *Molecular
Approaches to Developmental Biology*, pp. 415-424.).
Although not full length, the cDNA comprised most of
the coding sequence for the sunflower 2S albumin.
15 Northern and dot blot analysis indicated that this
gene is exclusively expressed in developing sunflower
seeds. Albumin transcripts and protein are first
detected 5 DPF, a full two days earlier than
helianthinin (11S), and reach maximal prevalence
around 12-15 DPF (Allen et al. 1987a).
20

25

30

35

-33-

EXAMPLE 6

1 ISOLATION OF A SUNFLOWER ALBUMIN 5' REGULATORY REGION

5 Genomic clones were isolated by screening a sunflower genomic DNA library using the Ha5 cDNA as a probe. Four independent genomic clones were shown to be identical by restriction enzyme digestion. Therefore, one clone (HaG5) was chosen for more detailed analysis.

10 A 2.3kb EcoRI/DraI fragment was sequenced (Allen et al., 1987b *Mol. Gen. Genet.* 210: 211-218). The HaG5 albumin gene contains two exons. The first exon (exon 1) is 575 nucleotides in length and the second exon (exon 2) is 310 nucleotides in length. A 190 nucleotide intron separates the two exons.
15 Nuclease protection experiments showed that the transcription start site was located 30 nucleotides upstream of the translational start site. (Allen et al 1987b, Fig. 2). Southern analysis of genomic DNA and the fact that only one gene was isolated in an
20 exhaustive screen indicated that HaG5 is a single copy gene in the sunflower genome.

An 889 bp upstream regulatory region (-860 to +29 of Figure 4; SEQ ID NO:2) was cloned in several steps from HaG5. A 1.1 kb EcoRI fragment was
25 subcloned in PBluescript™ (Stratagene) yielding pHaG5RI. PCR was performed on pHaG5RI with primers that resulted in the albumin 5' regulatory region being flanked by EcoRI and BamHI sites at the 5' and 3' ends, respectively. The restriction fragment was
30 cloned into the EcoRI/BamHI sites of pBluescript™

-34-

yielding pHaG5EB. Individual clones were sequenced to
1 check possible PCR mutations as well as the
orientation of their inserts. The sequence of the
albumin 5' regulatory region is shown in Fig. 4 (SEQ.
ID NO:2). The SalI/BamHI fragment of this construct
5 was excised and cloned into pAN3 (the parental borage
 $\Delta 6$ -desaturase containing plasmid), yielding pAN4. A
map of pAN4 and the intermediate vectors involved in
its construction are shown in Fig. 5. pAN1 is
described in Example 2. pBI101.1 is described in
10 (Jefferson et al. 1987 *EMBO J.* 6:3901-3907).

15

20

25

30

35

-35-

EXAMPLE 7

1 EXPRESSION OF $\Delta 6$ -DESATURASE UNDER CONTROL OF THE
SUNFLOWER ALBUMIN 5' REGULATORY REGION

The albumin 5' regulatory region was used to
5 drive the expression of a borage $\Delta 6$ -desaturase gene in
Arabidopsis. pAN4 was used to transform *Arabidopsis*
using the vacuum infiltration method of Bechtold et
al. 1993 *C.R. Acad. Sci. Paris* 316: 1194-1199. Levels
of $\Delta 6$ -desaturase activity were monitored by assaying
10 the corresponding fatty acid methyl esters of its
reaction products, γ -linolenic acid (GLA) and
octadecatetraenoic acid (OTA) using the methods
described in Example 3. GLA and OTA levels in
transgenic seeds ranged up to 10.2% (average of 4.4%)
15 and 3.6% (average of 1.7%), respectively, of the C18
fatty acids. No GLA or OTA was detected in the leaves
of these plants. In comparison, 35S promoter/ $\Delta 6$ -
desaturase transgenic plants produced GLA levels of up
20 to 3.1% of C18 fatty acids (average of 1.3%) in leaves
and no measurable OTA in seeds. These data are
summarized in Table 2.

25

30

35

-36-

TABLE 2
EXPRESSION OF THE BORAGE Δ^6 -DESATURASE IN TRANSGENIC PLANTS

PROMOTER	PLANT	SEED			LEAF		
		GLA* RANGE	OTA*		GLA RANGE	RANGE	OTA
Cauliflower mosaic virus 35S	tobacco	1.3	0.7-3.1	n.d.	20	19-22	9.7
Sunflower albumin	Arabidopsis	4.4 0.63- 3.6	3.1-10.2	1.7	8-11	n.d.	n.d.

*mean value expressed as the percent of the C₁₈ fatty acids n.d. not detected

1
5
10
15
20
25
30
35

EXAMPLE 8

1 Transformation of Oilseed Rape With an Expression
Cassette Which Comprises the Albumin 5' Regulatory
Region Linked to the Borage Delta 6-Desaturase Gene

Oilseed rape, Cv. Westar, was transformed
5 with the strain of *Agrobacterium tumefaciens* EHA105
containing the plasmid pAN4 (i.e. the borage Δ6-
desaturase gene under the control of the sunflower
albumin promoter-Example 6).

Terminal internodes of Westar were co-
10 cultivated for 2-3 days with induced *Agrobacterium*
tumefaciens strain EHA105 (Alt-Moerbe et al. 1988 *Mol.*
Genet. 213:1-8; James et al. 1993 *Plant Cell*
Reports 12:559-563), then transferred onto
regeneration medium (Boulter et al. 1990 *Plant Science*
15 70:91-99; Fry et al. 1987 *Plant Cell Reports* 6:321-
325). The regenerated shoots were transferred to
growth medium (Pelletier et al. 1983 *Mol.Gen. Menet.*
191:244-250), and a polymerase chain reaction (PCR)
test was performed on leaf fragments to assess the
20 presence of the gene.

DNA was isolated from the leaves according
to the protocol of K.M. Haymes et al. (1996) *Plant*
Molecular Biology Reporter, 14(3):280-284, and
resuspended in 100μl of water, without RNase
25 treatment. 5μl of extract were used for the PCR
reaction, in a final volume of 50μl. The reaction was
performed in a Perkin-Elmer 9600 thermocycler, with
the following cycles:

-38-

1 cycle: 95°C, 5 minutes
1 30 cycles: 95°C, 45 sec; 52°C, 45 sec
72°C, 1 minute
1 cycle: 72°C, 5 minutes

5 and the following primers (derived from near the metal box regions, as indicated in Fig. 1, SEQ. NO.:1):
5' TGG AAA TGG AAC CAT AA 3'
5' GGA AAC AAA TGA TGC TC 3'
Amplification of the DNA revealed the expected 549
10 base pair PCR fragment (Figure 7).
The positive shoots were transferred to elongation medium, then to rooting medium (DeBlock et al 1989 *Plant Physiol.* 91:694-701). Shoots with a well-developed root system were transferred to the
15 greenhouse. When plants were well developed, leaves were collected for Southern analysis in order to assess gene copy number.
Genomic DNA was extracted according to the procedure of Bouchez et al. (1996) *Plant Molecular Biology Reporter* 14:115-123, digested with the restriction enzymes *Bgl* I and/or *Cla* I, electrophoretically separated on agarose gel (Maniatis et al. 1982, in *Molecular Cloning; a Laboratory manual*. *Cold Spring Harbor Laboratory Press, Cold*
20 *Spring Harbor/NY*), and prepared for transfer to nylon membranes (Nytran membrane, Schleicher & Schuell) according to the instructions of the manufacturer. DNA was then transferred to membranes overnight by capillary action using 20XSSC (Maniatis et al. 1982).
25 30 Following transfer, the membranes were crosslinked by

- 39 -

UV (Stratagene) for 30 seconds and pre-hybridized for
1 hour at 65°C in 15 ml of a solution containing
6XSSC, 0.5%SDS and 2.25% w/w dehydrated skim milk in
glass vials in hybridization oven (Appligene). The
membranes were hybridized overnight in the same
5 solution containing a denatured hybridization probe
radiolabelled with ^{32}P to a specific activity of 10^8
cpm/ μg by the random primer method (with the Ready-To-
Go kit obtained from Pharmacia). The probe represents
a PCR fragment of the borage delta 6-desaturase gene
10 (obtained in the conditions and with the primers
detailed above). After hybridization, the filters
were washed at 65°C in 2XSSC, 0.1% SDS for 15 minutes,
and 0.2XSSC, 0.1%SDS for 15 minutes. The membranes
were then wrapped in Saran-Wrap and exposed to Kodak
15 XAR film using an intensifying screen at -70°C in a
light-proof cassette. Exposure time is generally 3
days.

The results obtained confirm the presence of
the gene. According to the gene construct, the number
20 of bands in each lane of DNA digested by *Bgl* I or *Cla*
I represents the number of delta 6-desaturase genes
present in the genomic DNA of the plant. The
digestion with *Bgl* I and *Cla* I together generates a
fragment of 3058 bp.

25 The term "comprises" or "comprising" is
defined as specifying the presence of the stated features,
integers, steps, or components as referred to in the
claims, but does not preclude the presence or addition
of one or more other features, integers, steps,
30 components, or groups thereof.

-40-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Rhone Poulen Agro
Thomas, Terry L.
Nunberg, Andrew N.
Beremand, Phillip D.

(ii) TITLE OF INVENTION: A SUNFLOWER ALBUMIN 5' REGULATORY REGION
FOR THE MODIFICATION OF PLANT SEED LIPID
COMPOSITION

(iii) NUMBER OF SEQUENCES: 22

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Scully, Scott, Murphy & Presser
(B) STREET: 400 Garden City Plaza
(C) CITY: Garden City
(D) STATE: New York
(E) COUNTRY: USA
(F) ZIP: 11530

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: 08/831,570
(B) FILING DATE: 09-APR-1997
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: DiGiglio, Frank S.
(B) REGISTRATION NUMBER: 31,346
(C) REFERENCE/DOCKET NUMBER: 10545

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (516) 742-4343
(B) TELEFAX: (516) 743-4366

-41-

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1684 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 43..1387

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATATCTGCCT ACCCTCCCAA AGAGAGTAGT CATTTTCAT CA ATG GCT GCT CAA Ile Lys Lys Tyr Ile Thr Ser Asp Glu Leu Lys Asn His Asp Lys Pro	54
5 10 15 20	
GGA GAT CTA TGG ATC TCG ATT CAA GGG AAA GCC TAT GAT GTT TCG GAT Gly Asp Leu Trp Ile Ser Ile Gln Gly Lys Ala Tyr Asp Val Ser Asp	102
25 30 35	
TGG GTG AAA GAC CAT CCA GGT GGC AGC TTT CCC TTG AAG AGT CTT GCT Trp Val Lys Asp His Pro Gly Ser Phe Pro Leu Lys Ser Leu Ala	150
40 45 50	
GGT CAA GAG GTA ACT GAT GCA TTT GTT GCA TTC CAT CCT GCC TCT ACA Gly Gln Glu Val Thr Asp Ala Phe Val Ala Phe His Pro Ala Ser Thr	198
55 60 65	
TGG AAG AAT CTT GAT AAG TTT TTC ACT GGG TAT TAT CTT AAA GAT TAC Trp Lys Asn Leu Asp Lys Phe Phe Thr Gly Tyr Tyr Leu Lys Asp Tyr	246
70 75 80	
TCT GTT TCT GAG GTT TCT AAA GAT TAT AGG AAG CTT GTG TTT GAG TTT Ser Val Ser Glu Val Ser Lys Asp Tyr Arg Lys Leu Val Phe Glu Phe	294
85 90 95 100	
TCT AAA ATG GGT TTG TAT GAC AAA AAA GGT CAT ATT ATG TTT GCA ACT Ser Lys Met Gly Leu Tyr Asp Lys Lys Gly His Ile Met Phe Ala Thr	342
105 110 115	

-42-

TTG TGC TTT ATA GCA ATG CTG TTT GCT ATG AGT GTT TAT GGG GTT TTG Leu Cys Phe Ile Ala Met Leu Phe Ala Met Ser Val Tyr Gly Val Leu 120 125 130	438
TTT TGT GAG GGT GTT TTG GTA CAT TTG TTT TCT GGG TGT TTG ATG GGG Phe Cys Glu Gly Val Leu Val His Leu Phe Ser Gly Cys Leu Met Gly 135 140 145	486
TTT CTT TGG ATT CAG AGT GGT TGG ATT GGA CAT GAT GCT GGG CAT TAT Phe Leu Trp Ile Gln Ser Gly Trp Ile Gly His Asp Ala Gly His Tyr 150 155 160	534
ATG GTA GTG TCT GAT TCA AGG CTT AAT AAG TTT ATG GGT ATT TTT GCT Met Val Val Ser Asp Ser Arg Leu Asn Lys Phe Met Gly Ile Phe Ala 165 170 175 180	582
GCA AAT TGT CTT TCA GGA ATA AGT ATT GGT TGG TGG AAA TGG AAC CAT Ala Asn Cys Leu Ser Gly Ile Ser Ile Gly Trp Trp Lys Trp Asn His 185 190 195	630
AAT GCA CAT CAC ATT GCC TGT AAT AGC CTT GAA TAT GAC CCT GAT TTA Asn Ala His His Ile Ala Cys Asn Ser Leu Glu Tyr Asp Pro Asp Leu 200 205 210	678
CAA TAT ATA CCA TTC CTT GTT GTG TCT TCC AAG TTT TTT GGT TCA CTC Gin Tyr Ile Pro Phe Leu Val Val Ser Ser Lys Phe Phe Gly Ser Leu 215 220 225	726
ACC TCT CAT TTC TAT GAG AAA AGG TTG ACT TTT GAC TCT TTA TCA AGA Thr Ser His Phe Tyr Glu Lys Arg Leu Thr Phe Asp Ser Leu Ser Arg 230 235 240	774
TTC TTT GTA AGT TAT CAA CAT TGG ACA TTT TAC CCT ATT ATG TGT GCT Phe Phe Val Ser Tyr Gln His Trp Thr Phe Tyr Pro Ile Met Cys Ala 245 250 255 260	822
GCT AGG CTC AAT ATG TAT GTA CAA TCT CTC ATA ATG TTG TTG ACC AAG Ala Arg Leu Asn Met Tyr Val Gln Ser Leu Ile Met Leu Leu Thr Lys 265 270 275	870
AGA AAT GTG TCC TAT CGA GCT CAG GAA CTC TTG GGA TGC CTA GTG TTC Arg Asn Val Ser Tyr Arg Ala Gln Glu Leu Leu Gly Cys Leu Val Phe 280 285 290	918
TCG ATT TGG TAC CCG TTG CTT GTT TCT TGT TTG CCT AAT TGG GGT GAA Ser Ile Trp Tyr Pro Leu Leu Val Ser Cys Leu Pro Asn Trp Gly Glu 295 300 305	966
AGA ATT ATG TTT GTT ATT GCA AGT TTA TCA GTG ACT GGA ATG CAA CAA Arg Ile Met Phe Val Ile Ala Ser Leu Ser Val Thr Gly Met Gln Gln 310 315 320	1014

-43-

GTT CAG TTC TCC TTG AAC CAC TTC TCT TCA AGT GTT TAT GTT GGA AAG Val Gln Phe Ser Leu Asn His Phe Ser Ser Val Tyr Val Gly Lys 325 330 335 340	1062
CCT AAA GGG AAT AAT TGG TTT GAG AAA CAA ACG GAT GGG ACA CTT GAC Pro Lys Gly Asn Asn Trp Phe Glu Lys Gln Thr Asp Gly Thr Leu Asp 345 350 355	1110
ATT TCT TGT CCT CCT TGG ATG GAT TGG TTT CAT GGT GGA TTG CAA TTC Ile Ser Cys Pro Pro Trp Met Asp Trp Phe His Gly Gly Leu Gln Phe 360 365 370	1158
CAA ATT GAG CAT CAT TTG TTT CCC AAG ATG CCT AGA TGC AAC CTT AGG Gln Ile Glu His His Leu Phe Pro Lys Met Pro Arg Cys Asn Leu Arg 375 380 385	1206
AAA ATC TCG CCC TAC GTG ATC GAG TTA TGC AAG AAA CAT AAT TTG CCT Lys Ile Ser Pro Tyr Val Ile Glu Leu Cys Lys Lys His Asn Leu Pro 390 395 400	1254
TAC AAT TAT GCA TCT TTC TCC AAG GCC AAT GAA ATG ACA CTC AGA ACA Tyr Asn Tyr Ala Ser Phe Ser Lys Ala Asn Glu Met Thr Leu Arg Thr 405 410 415 420	1302
TTG AGG AAC ACA GCA TTG CAG GCT AGG GAT ATA ACC AAG CCG CTC CCG Leu Arg Asn Thr Ala Leu Gln Ala Arg Asp Ile Thr Lys Pro Leu Pro 425 430 435	1350
AAG AAT TTG GTA TGG GAA GCT CTT CAC ACT CAT GGT T AAAATTACCC Lys Asn Leu Val Trp Glu Ala Leu His Thr His Gly 440 445	1397
TTAGTTCATG TAATAATTG AGATTATGTA TCTCCTATGT TTGTGTCTTG TCTTGGTTCT ACTTGTGGA GTCATGGAA CTTGCTTTT ATGGTTTATT AGATGTTTT TAATATATT TAGAGGTTTT GCTTCATCT CCATTATTGA TGAATAAGGA GTTGCATATT GTCAATTGTT GTGCTCAATA TCTGATATTT TGGAAATGTAC TTTGTACCAC GTGGTTTCA GTTGAAGCTC ATGTGTACTT CTATAGACTT TGTTTAAATG GTTATGTCAT GTTATTT	1457 1517 1577 1637 1684

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 843 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

-44-

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GAATTCTATC ACTAGTGACC ACCCCATCCC CTTATTCAA TAATGGAACA CAAAAAAATT	60
TTAAAAAATA GTTGCTGTTA ATTGTTAAC CGTCATTTTC CAACATTACT AGCTAATCGT	120
TAATTGATCT TCATAAAAAA AAAAATTGCT ATGGGTACTA TTGAGATTGT ATATCTTATC	180
AGTTAGGCCT AAGGGGGGGG TCAGTGATAT TACGAATGAT ACAAACATCA ACGCGTGGAA	240
CATTACAAA TTCCGTAATT TTTTCAACGC CGTGATGGTT TTTTTTTTTT TTTTTTTTTT	300
TGATGGTAAT TGTTGGTTGG GGGGAAATTA TTGGGTATGG TGTTGAGTAT GACCACCCCC	360
ACTAAAAAAG GTTGTGAGTG ATGTAAAAT GGTTGCTGAC ATGACGAAAC ATAATTGGAT	420
ATTGTGAGTG ATAAAATTTC ATCATTAGTG ACCACCCCGC CTCCCCTTAT CATATGTTGT	480
TATCTTCCAT AGTTGCGGTA TACCAACATA TGGTAGTTTT TATATTATA GTTTATATTT	540
TCATTAAACT CTCTCGCCA GGCTACTTGT ATTGTAATCA TATGGAATCT CAACTCCAGT	600
TGGAGCCATT CCATCATATA TTTCCATTTC CAAACAAAGA GAATTGACAC CTCATACATA	660
CTCCAAAGCA TACTCCACT TGCTATAATT TTCATGTAAA AACTCGTACG TGTTATTGCA	720
CAATGTTCAT ATAACGCCAC CGATTAAACT CACCTCTCCA CGTATGAACC TCCACCCACC	780
ATATATACGC ACCACCAACCA CACCATAATT CACACAACCA CAACACCATC TCCCACAGGA	840
TCC	843

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Trp Ile Gly His Asp Ala Gly His
1 5

-45-

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Asn Val Gly His Asp Ala Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Val Leu Gly His Asp Cys Gly His
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Val Ile Ala His Glu Cys Gly His
1 5

-46-

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Val Ile Gly His Asp Cys Ala His
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Val Val Gly His Asp Cys Gly His
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

His Asn Ala His His
1 5

-47-

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

His Asn Tyr Leu His His
1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

His Arg Thr His His
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

His Arg Arg His His
1 5

-48-

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

His Asp Arg His His
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

His Asp Gln His His
1 5

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

His Asp His His His
1 5

-49-

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

His Asn His His His
1 5

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Phe Gln Ile Glu His His
1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

His Gln Val Thr His His
1 5

-50-

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

His Gln Val Thr His His
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

His Val Ala His His
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

His Ile Pro His His
1 5

-51-

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

His Val Pro His His
1 5

- 52 -

What is claimed is:

1

1. An isolated nucleic acid encoding an albumin 5' regulatory region which directs seed-specific expression selected from the group consisting of the nucleotide sequence set forth in SEQ ID NO:2, the nucleotide sequence set forth in SEQ ID NO:2 having an insertion, deletion, or substitution of one or more nucleotides, and a contiguous fragment of the nucleotide sequence set forth in SEQ ID NO:2.

10

2. An expression cassette which comprises the albumin 5' regulatory region of Claim 1 operably linked to a heterologous gene.

15 3. The expression cassette of Claim 2 wherein the heterologous gene is at least one of a fatty acid synthesis gene or a lipid metabolism gene.

20 4. The expression cassette of Claim 3 wherein the heterologous gene is selected from the group consisting of a lipid desaturase gene, an acyl carrier protein (ACP) gene, a thioesterase gene, an acetyl transacylase gene, an acetyl-coA carboxylase gene, a ketoacyl synthase gene, a malonyl transacylase gene or an elongase gene.

25 5. The expression cassette of Claim 4 wherein the lipid desaturase gene is selected from the group consisting of a Δ6-desaturase gene, a Δ12-desaturase gene, and a Δ15-desaturase gene.

6. An expression vector which comprises the expression cassette of any one of Claims 2-5.

30 7. A cell comprising the expression cassette of any one of Claims 2-5.

-53-

8. A cell comprising the expression vector
1 of Claim 6.

9. The cell of Claim 7 wherein said cell is
a bacterial cell or a plant cell.

10. The cell of Claim 8 wherein said cell
5 is a bacterial cell or a plant cell.

11. A transgenic plant comprising the
expression cassette of any one of Claims 2-5.

12. A transgenic plant comprising the
expression vector of Claim 6.

10 13. A plant which has been regenerated from
the plant cell of Claim 9.

14. A plant which has been regenerated from
the plant cell of Claim 10.

15 15. The plant of Claim 12 or 13 wherein
said plant is at least one of a sunflower, soybean,
maize, cotton, tobacco, peanut, oil seed rape or
Arabidopsis plant.

16. Progeny of the plant of Claim 11 or 12.

17. Seed from the plant of Claim 11 or 12.

20 18. A method of producing a plant with
increased levels of a product of a lipid metabolism
gene which comprises:

25 (a) transforming a plant cell with an
expression vector comprising the isolated nucleic acid
of Claim 1 operably linked to at least one of an
isolated nucleic acid coding for a fatty acid
synthesis gene or a lipid metabolism gene; and

(b) regenerating a plant with increased
levels of the product of said fatty acid synthesis

30

- 54 -

gene or said lipid metabolism gene from said plant
1 cell.

19. A method of producing a plant with increased levels of gamma linolenic acid (GLA) content which comprises:

5 (a) transforming a plant cell with an expression vector comprising the isolated nucleic acid of Claim 1 operably linked to a $\Delta 6$ -desaturase gene; and

10 (b) regenerating a plant with increased levels of GLA from said plant cell.

20. The method of Claim 19 wherein said $\Delta 6$ -desaturase gene is at least one of a cyanobacterial $\Delta 6$ -desaturase gene or a borage $\Delta 6$ -desaturase gene.

21. The method of Claim 18 or 19 wherein 15 said plant is a sunflower, soybean, maize, tobacco, cotton, peanut, oil seed rape or *Arabidopsis* plant.

22. The method of Claim 18 wherein said fatty acid synthesis gene or said lipid metabolism gene is at least one of a lipid desaturase, an acyl 20 carrier protein (ACP) gene, a thioesterase gene an elongase gene, an acetyl transacylase gene, an acetyl-coA carboxylase gene, a ketoacyl synthase gene, or a malonyl transacylase gene.

23. A method of inducing production of at 25 least one of gamma linolenic acid (GLA) or octadecatetraenoic acid (OTA) in a plant deficient or lacking in GLA which comprises transforming said plant with an expression vector comprising an the isolated nucleic acid of Claim 1 operably linked to a $\Delta 6$ -

30

- 55 -

desaturase gene and regenerating a plant with
1 increased levels of at least one of GLA or OTA.

24. A method of decreasing production of a fatty acid synthesis or lipid metabolism gene in a plant which comprises:

5 (a) transforming a plant cell with an expression vector comprising the isolated nucleic acid of Claim 1 operably linked to a nucleic acid sequence complementary to a fatty acid synthesis or lipid metabolism gene; and
10 (b) regenerating a plant with decreased production of said fatty acid synthesis or said lipid metabolism gene.

25. A method of cosuppressing a native fatty acid synthesis or lipid metabolism gene in a
15 plant which comprises:

(a) transforming a cell of the plant with an expression vector comprising the isolated nucleic acid of Claim 1 operably linked to a nucleic acid sequence encoding a fatty acid synthesis or lipid metabolism
20 gene native to the plant; and
(b) regenerating a plant with decreased production of said fatty acid synthesis or said lipid metabolism gene.

25

30

35

FIGURE 1

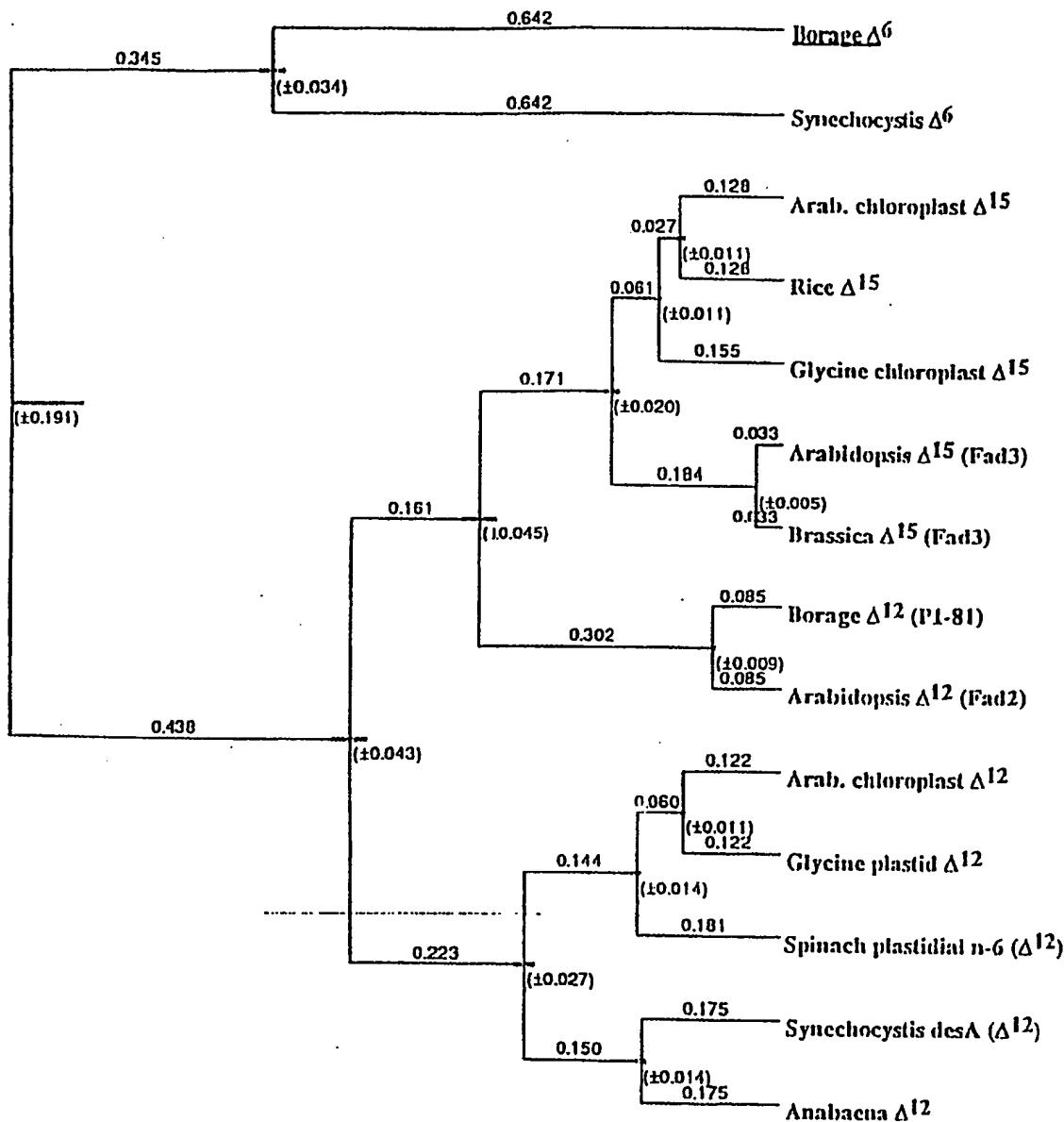


FIGURE 2

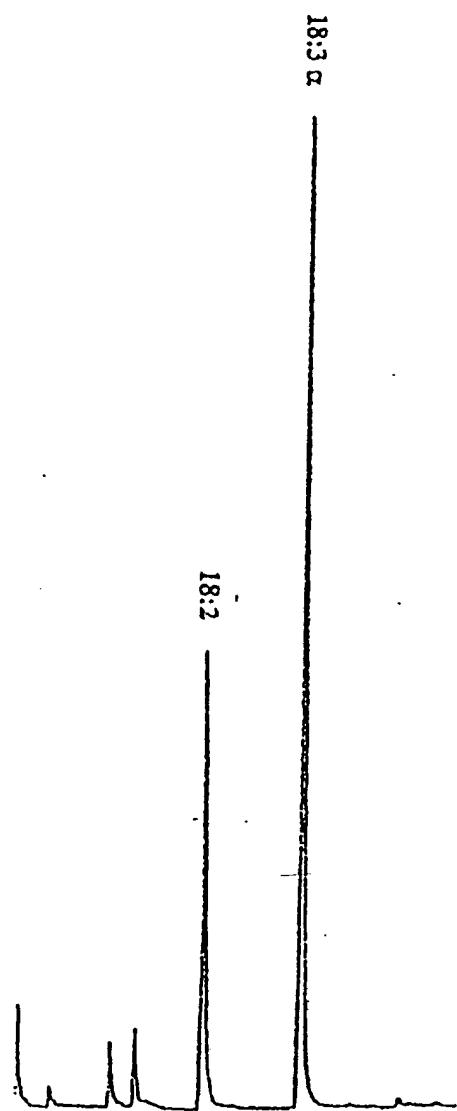


FIGURE 3A

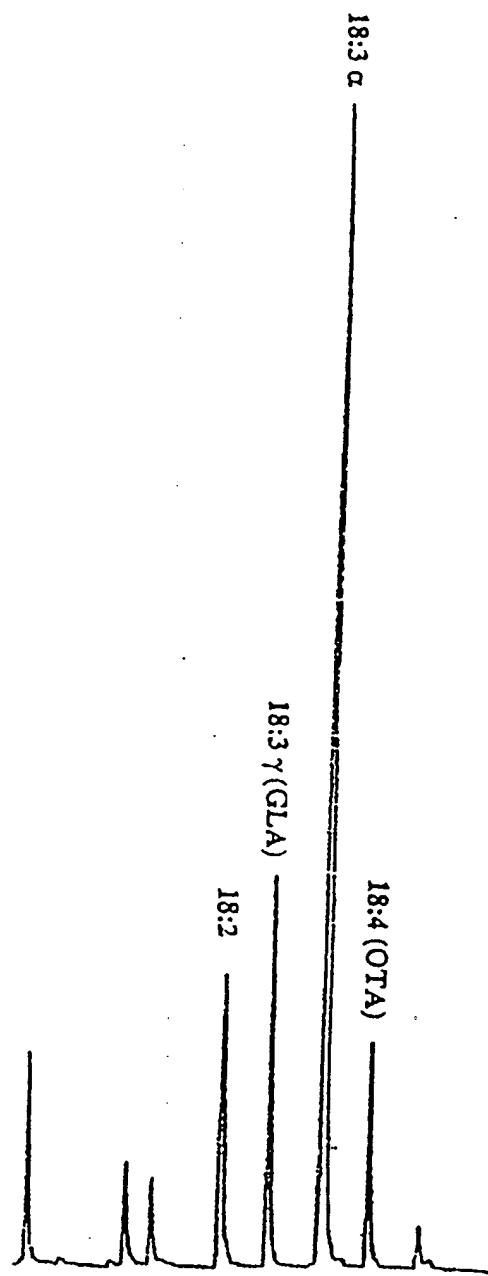


FIGURE 3B

GAACTCTAAC ACTAGTGACC ACGCCATGCC CTTATTCAAA TAAAGGAACA	-811
CGAAAGAAAT TTAAAGAAAT AGTTCGCTGT AAATGTTAA CGGTCAATTG	-761
CCAACATTAC TAGCTAATCG TTATTTGATC TTCAATAAAA AAAAATTCGC	-711
TAAAGGGTACT ATATGAGATIG TATATCTTAT CAGTTAGGCC TAAAGGGGGCG	-661
GTCAGTGATA TTACGAATGA TACAAACATC AACCGGIGGA ACATTTACAAA	-611
TTCCTATCCC CACCTCCANG TATAACGGGT GTTGTGTCGA CGGTTTGATG	-561
ATTCGGTAAT TTTCCTAACG CGTGTGATGGT TTTTTTTTTT TTTTTTTTGT	-511
TTGATGGTAA TIGTTGGITG GGGGGAAATG ATGGGTATG GIGTTGAGTG	-461
ATGACCAACCC CCACAAAGGTTGAG TGATGTAAGG ATGGTTGCTG	-411
ACATGACGAA ACATTAATGG ATATGIGAG TGATTAATTT TTATCTTATAG	-361
TGACCCACCCCC GCCCTCCCCCT ATCTTATGTT GTTACCTTCC ATAGTGGGG	-311
TATACCAACA TATGGTATGTT TTATATTTA TAGTTTATAT TTTCATTAAG	-261
CCTCTTGGC CAGGCTACTT GTATGTAAT CATACTGGAT CTCAACTOCA	-211
CTTGGAGCCA TCCCTCATTA TATTCCATT TCCAAACAAA GAGATGAC	-161
ACCCTATACA TACCTCAAAG CATACTCCA CTGCTATAA TTCTCAATGTA	-111
AAAACCTGTA CGTGTATTC GACAAAGTTC ATATAACGCC ACCGTTAAA	-61
CCTACCCCTC CACGTATGAA CCTCCACCCCA CCATATATAC GCACCAACAC	-11
CACACCAAAA TCTCACACAAAC CACAAACACCA TCTCCACAG <u>GAICC</u>	-29

FIGURE 4

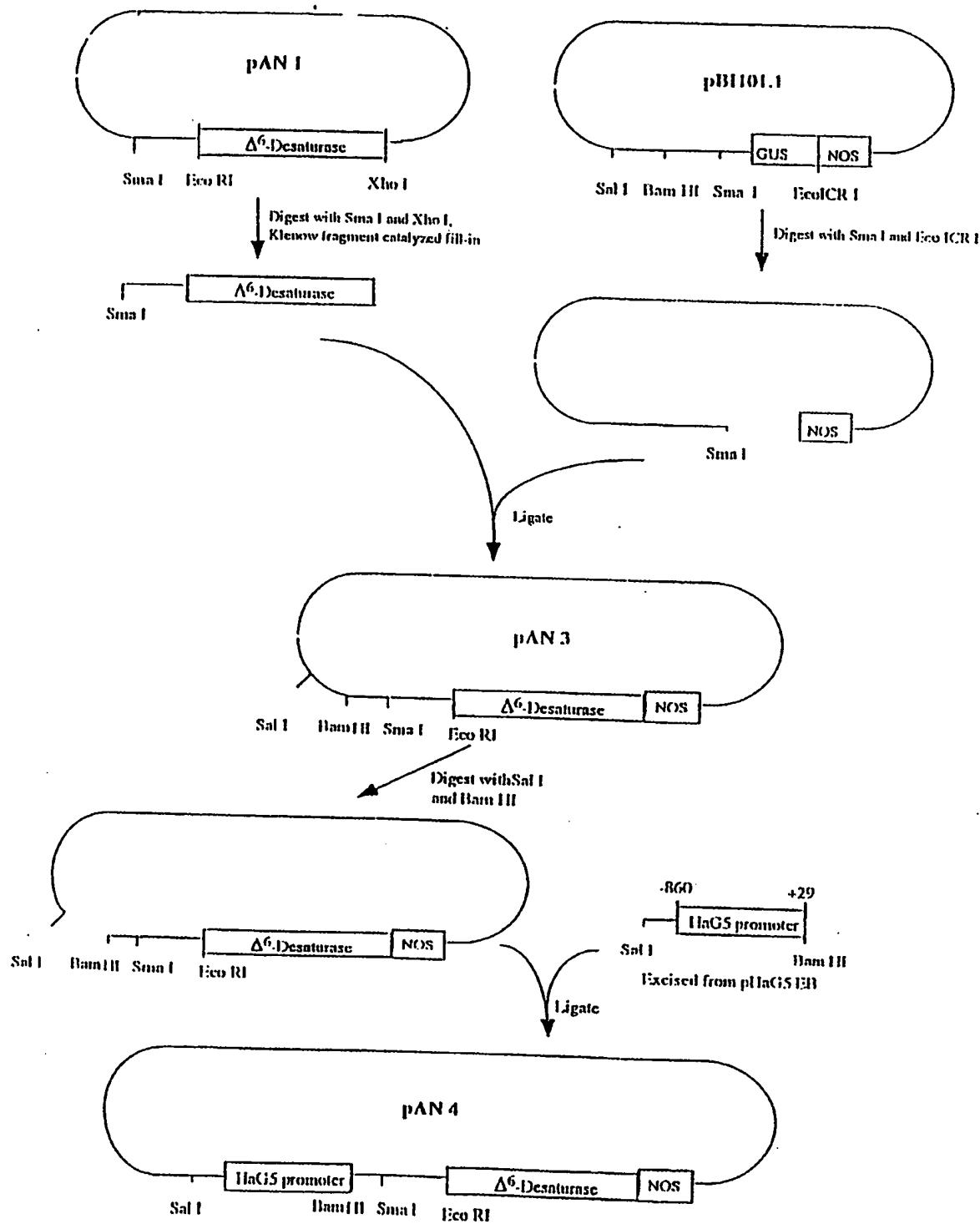
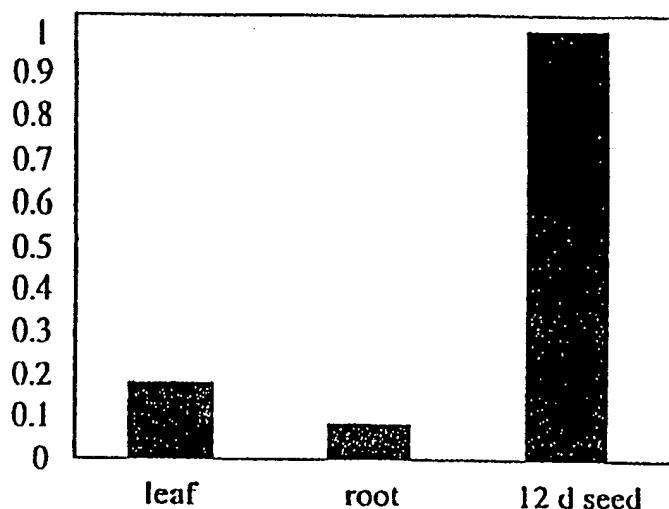


FIGURE 6B



Borage tissue



FIGURE 6A

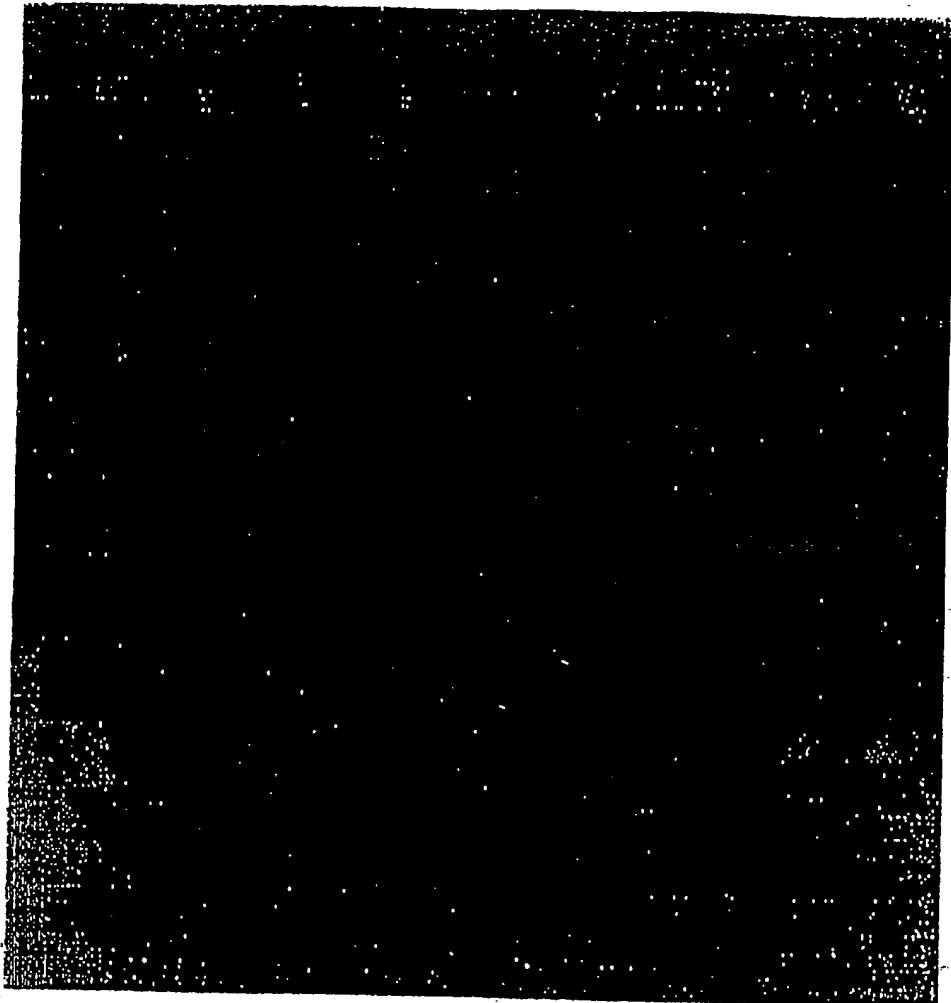
BEST AVAILABLE COPY

WO 98/45460

7/7

PCT/US98/07178

FIGURE 7



BEST AVAILABLE COPY

INTERNATIONAL SEARCH REPORT

Intern'l Application No
PCT/US 98/07178

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/29 C12N15/53 A01H5/00 A01H5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALLEN, R.D., ET AL.: "Sequence and expression of a gene encoding an albumin storage protein in sunflower" MOL. GEN. GENET., vol. 210, 1987, pages 211-218, XP002074856 see the whole document ---	1
Y	WO 96 21022 A (RHONE POULENC AGROCHIMIE) 11 July 1996 see page 14, line 3 - page 15, line 2; examples 6,11,13,14 ---	2-23
Y	WO 92 17580 A (RHONE POULENC AGROCHIMIE) 15 October 1992 see page 20, line 1 - page 11 ---	2-23
A	WO 94 10189 A (CALGENE INC) 11 May 1994 see examples 3,4 ---	1-25
A	-/-	1-4

Further documents are listed in continuation of box O.

Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

4 September 1998

Date of mailing of the international search report

07.09.98

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl
Fax: (+31-70) 340-3016

Authorized officer

Maddox, A

INTERNATIONAL SEARCH REPORT

Intern'l Application No
PCT/US 98/07178

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 91 13972 A (CALGENE INC) 19 September 1991 see examples 9,13 ---	1-4
A	WO 92 11373 A (DU PONT) 9 July 1992 see page 67, line 25 - page 68, line 13 ---	1-4
A	WO 93 11245 A (DU PONT) 10 June 1993 see examples 10-13 ---	1-5,24
A	WO 94 18337 A (MONSANTO CO ;UNIV MICHIGAN (US); GIBSON SUSAN IRMA (US); KISHORE G) 18 August 1994. see the whole document ---	1,3-5, 24,25
A	WO 94 11516 A (DU PONT ;LIGHTNER JONATHAN EDWARD (US); OKULEY JOHN JOSEPH (US)) 26 May 1994 see page 98 - page 110 ---	1,3-5,24
A	WO 96 06936 A (DU PONT ;HITZ WILLIAM DEAN (US)) 7 March 1996 see page 48, line 23 - page 53, line 10 ---	1-5,24, 25
A	BEREMAND, P.D., ET AL.: "Production of gamma-linolenic acid by transgenic plants expressing cyanobacterial or plant delta6-desaturase genes" PHYSIOL., BIOCHEM. MOL. BIOL. PLANT LIPIDS, [PROC. INT. SYMP. PLANT LIPIDS], 12TH (1997), 351-353. EDITOR(S): WILLIAMS, JOHN PETER; KHAN, MOBASHHER UDDIN; LEM, NORA WAN. PUBLISHER: KLUWER, DORDRECHT, NETH. CODEN: 65BHAZ, XP002076486 see page 353	1-23
0,A	& BEREMAND, P.D. ET AL.: DISCLOSURE AT THE 12TH INTERNATIONAL SYMPOSIUM ON PLANT LIPIDS, HELD JULY 7-12, 1996, UNIVERSITY OF TORONTO, TORONTO, ---	1-23
A	TOEPFER R ET AL: "MODIFICATION OF PLANT LIPID SYNTHESIS" SCIENCE, vol. 268, 5 May 1995, pages 681-685, XP002014017 see the whole document ---	24,25
A	GIBSON, S., ET AL.: "Cloning of a temperature-regulated gene encoding a chloroplast omega-3 desaturase from Arabidopsis thaliana" PLANT PHYSIOLOGY, vol. 104, 1994, pages 1615-1621, XP002075189 see the whole document -----	25

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/07178

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9621022	A 11-07-1996	US 5614393 A		25-03-1997
		AU 4673596 A		24-07-1996
		CA 2207906 A		11-07-1996
		CN 1177379 A		25-03-1998
		EP 0801680 A		22-10-1997
		US 5789220 A		04-08-1998
-----	-----	-----	-----	-----
WO 9217580	A 15-10-1992	AU 672949 B		24-10-1996
		AU 1681992 A		02-11-1992
		BR 9205873 A		23-08-1994
		EP 0579726 A		26-01-1994
		JP 6509939 T		10-11-1994
		MX 9201592 A		01-10-1992
-----	-----	-----	-----	-----
WO 9410189	A 11-05-1994	CA 2148358 A		11-05-1994
		EP 0666865 A		16-08-1995
		JP 8502891 T		02-04-1996
-----	-----	-----	-----	-----
WO 9113972	A 19-09-1991	EP 0472722 A		04-03-1992
		US 5723595 A		03-03-1998
-----	-----	-----	-----	-----
WO 9211373	A 09-07-1992	AU 662506 B		07-09-1995
		AU 9116191 A		22-07-1992
		DE 69113635 D		09-11-1995
		DE 69113635 T		13-06-1996
		DK 563191 T		04-12-1995
		EP 0563191 A		06-10-1993
		US 5530186 A		25-06-1996
-----	-----	-----	-----	-----
WO 9311245	A 10-06-1993	AU 675923 B		27-02-1997
		AU 3228893 A		28-06-1993
		CA 2124673 A		10-06-1993
		EP 0616644 A		28-09-1994
		JP 7501701 T		23-02-1995
-----	-----	-----	-----	-----
WO 9418337	A 18-08-1994	EP 0684998 A		06-12-1995
		JP 8506490 T		16-07-1996
-----	-----	-----	-----	-----
WO 9411516	A 26-05-1994	AU 5407594 A		08-06-1994
		CA 2149223 A		26-05-1994

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern	Application No
	PCT/US 98/07178

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9411516	A	EP	0668919 A	30-08-1995
		JP	8503364 T	16-04-1996
-----	-----	-----	-----	-----
WO 9606936	A	07-03-1996	AU 3410295 A BR 9509502 A CA 2198222 A EP 0778896 A HU 76842 A JP 10505237 T PL 319103 A	22-03-1996 30-09-1997 07-03-1996 18-06-1997 28-11-1997 26-05-1998 21-07-1997
-----	-----	-----	-----	-----

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.